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The G protein-coupled receptor GPR30 mediates the proliferative and invasive effects induced by hydroxytamoxifen in endometrial cancer cells

Gui-Qiang Du [a], Long Zhou [a], Xiao-Yue Chen [a], Xiao-Ping Wan [a,*], Yin-Yan He [b]

[a] Department of Obstetrics and Gynecology, The International Peace Maternity and Child Health Hospital of the China Welfare Institute Affiliated to Shanghai Jiao Tong University, 910, Hengshan Road, Shanghai, China
[b] Department of Obstetrics and Gynecology, Shanghai First People's Hospital, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

The selective ER modulator tamoxifen (TAM[1]) is the most widely used ER antagonist for treatment of women with hormone-dependent breast tumor. However, long-term treatment is associated with an increased risk of endometrial cancer. The aim of the present study was to demonstrate new insight into the role of G-protein coupled receptor 30 (GPR30) in the activity of TAM, which promoted endometrial cancer. In endometrial cancer cell lines ISHIKAWA and KLE, the potential of 4-hydroxytamoxifen (OHT), the active metabolite of TAM, 17β-estradiol (E2) and G1, a non-steroidal GPR30-specific agonist to promote cell proliferation and invasion was evaluated. All agents above induced high proliferative and invasive effects, while the down-regulation of GPR30 or the interruption of MAPK signal pathway partly or completely prevented the action of the regent. Moreover, the RNA and protein expression of GPR30 was up-regulated by G1, E2 or OHT in both cell lines. The present study provided a new insight into the mechanism involved in the agonistic activity exerted by TAM in the uterus.

1. Introduction

Estrogen is vital hormone, which regulates the growth and differentiation of many tissues, and is the one of the risk factors for endometrial cancer and breast tumors, which accounts for 40% of cancer among the women [1]. The estrogen binding to estrogen receptors (ER) α and β was the main mechanism responsible for the diverse effects [2]. Approximately 50% of all breast cancers demonstrated elevated levels of ER. Consequently, ERα antagonists such as tamoxifen (TAM) and raloxifene have been used clinically and demonstrated advantages in treating ERα-positive breast cancer [3,4]. Although primarily considered an anti-estrogen, TAM also exhibited some mild estrogenic effects in the uterus, bone, and the cardiovascular system [5]. Studies have suggested that incidence and severity of endometrial cancer increased in women treated with TAM [6]. TAM and its active metabolite 4-hydroxytamoxifen (OHT) stimulated the proliferation of uterine cell growth in vivo and cultured human endometrial carcinoma cells [7–9].

The G-protein coupled receptor 30 (GPR30) is a membrane-associated estrogen receptor, that might alter gene expression independently of the nuclear ERs [10]. GPR30 belongs to the seven-transmembrane GPCR family, which signals through heterotrimeric guanine nucleotide-binding proteins to alter the activity of effector proteins. Cellular activation by GPR30 occurs through a mechanism involving transactivation of epidermal growth factor receptors (EGFRs) via a G protein-dependent pathway and activation of mitogen activated protein kinase (MAPK), adenylyl cyclase, and phosphoinositide 3-kinase (PI3K) [10–12]. GPR30 acted in physiological processes such as the attenuation of liver injury and pubertal adrenal development [13,14], and mediated the proliferative effects of estrogen in a number of cancer cell lines [15,16]. Furthermore, it has been shown that 17β-estradiol (E2) and G1, a GPR30-specific ligand, stimulated the production and activity of matrix metalloproteinase (MMP), the proteolysis of which was involved in cancer invasion and metastasis, increased secretion of interleukin-6, the activation of which was associated with tumor growth, and mediated invasion and carcinogenesis in endometrial cancer cell line in a GPR30 dependent manner [17,18]. The classical ERα antagonists TAM also acted as GPR30 agonists [10,19].

To demonstrate the possible role of GPR30 in the mediating the signal of TAM in endometrial cancer cell, experiments and found E2, G1 and OHT promote the proliferation and invasion in a GPR30-dependent manner in ISHIKAWA and KLE human endometrial cancer cells. Moreover, GPR30 was also up-regulated by the

* Corresponding author.
E-mail address: wanxiaoping61@126.com (X.-P. Wan).

[1] *Abbreviations:* GPR30, G protein-coupled receptor 30; E2, 17β-estradiol; MMP, Matrix metal-loproteinase; MAPK, Mitogen-activated protein kinase; TAM, Tamoxifen; OHT, 4-Hydroxytamoxifen; ShGPR30-pGFP-V-RS, Short hairpin RNA (shRNA) constructs against GPR30 in pGFP-V-RS; shiv-pGFP-V-RS vector, HuSH 29-mer noneffective against enhanced GFP vector; EGFR, Epidermal growth factor receptor; ERK, Extracellular-signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium.

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same agents. The results showed insights into the mechanism through which OHT exerted an action in the endometrial cancer cells.

2. Materials and methods

2.1. Cell culture

The human endometrial cell line ISHIKAWA and KLE was obtained from the Chinese Academy of Sciences Committee Type Culture Collection cell bank. The cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (11030; Gibco, Auckland, New Zealand) supplemented with 10% fetal bovine serum (FBS) (16000-44; Gibco, Carlsbad, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

2.2. Stable transfection

One hundred thousand cells were plated into 24-well dishes with 500 μl regular growth medium per well on the day before transfection. According to the manufacturer's instructions, the cells were transfected with short hairpin RNA (shRNA) constructed against GPR30 in pGFP-V-RS (TG316565; OriGene Technologies, Rockville, MD) (Fig. 1A), and the HuSH 29-mer non-effective against enhanced GFP vector (pRS.TR30003; OriGene Technologies) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). To obtain a stable cell line, selection pressure was maintained with puromycin at a concentration of 0.5–1.0 μg/ml in the growth medium for two weeks. Clonal populations of cells were selected by transferring well-isolated single clumps of cells (the clonal ancestor and cells divided from it) into a six-well plate, and the growth of these cells were continued in the selection medium for two additional passages.

2.3. Real-time RT-PCR

Total RNA was extracted from the cells using TRI Reagent (TR118; Molecular Research Center, Cincinnati, OH). The cDNA was generated with oligo (dT) 18 primers using a Revert Aid First Strand cDNA Synthesis Kit (K1622; Fermentas Life Science, St. Leon-Rot, Germany). A 50 μl PCR amplification of single-strand cDNA was performed with 40 cycles of denaturation (94 °C) for 60 s, annealing (55 °C) for 30 s, and elongation (72 °C) for 30 s using PerfectShot Ex Taq (Loading Dye Mix) (DRR05TA; Takara, Dalian, China). The primer sequences were as mentioned in Supplementary material Table 1.

2.4. Proliferation assay

Cells were seeded in 96-well plates at 2×10^5 cells/ml and cultured for 24 h. The cells were then transferred to phenol red-free and serum-free medium (DMEM/F12, 11039; Gibco, Carlsbad, CA) and cultured for 24 h, followed by the addition of G1 (371705; Calbiochem, Darmstadt, Germany), E2 (E2758-250MG; Sigma Aldrich, St. Louis, MO) or OHT (H7904-5MG; Sigma Aldrich, St. Louis, MO) in the presence or absence of the MAPK inhibitor U0126 (30 μM) (9903; Cell Signaling Technology, Beverly, MA). The cells were further cultured with phenol red-free DMEM/F12 containing 5% dextran-coated charcoal-treated FBS (S181F; Biowest, Nuaillé, France). Cell proliferation was evaluated using a Cell Counting Kit-8 (CK04-11; Dojindo Molecular Technologies, Gaithersburg,

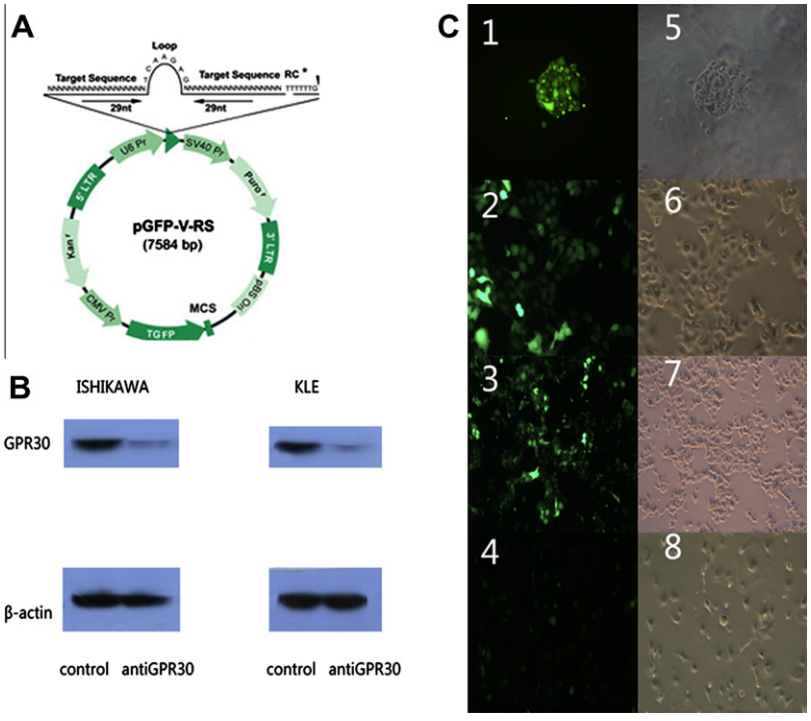

Fig. 1. Expression of GPR30 is stably down-regulated in KLE and ISHIKAWA cell lines. (A) The pGFP-V-RS plasmid vector pattern. The pGFP-V-RS plasmid vector was created with an integrated turboGFP element to readily verify transfection efficiency. It also incorporates both a kanamycin and puromycin resistance elements for greater selection capabilities .The shRNA expression cassette consists of a 29 nt GPR30-specific sequence, a 7 nt loop, and another 29 nt reverse complementary sequence, all under the control of the human U6 promoter. A termination sequence (TTTTTT) is located immediately downstream of the second 29 nt reverse complementary sequence to terminate the transcription by RNA Pol III. (B) The expression level of GPR30 protein in ISHIKWA and KLE cells transfected with ShGPR30-pGFP-V-RS (AntiGPR30) or shiv-pGFP-V-RS (Control) were detected using western-blot. (C) Cells were then visualized by fluorescence microscopy after stable transfection with pGFP-V-RS: ISHIKAWA cells transfected with ShGPR30-pGFP-V-RS under fluorescence (1) or white light (5); ISHIKAWA cells transfected with shiv-pGFP-V-RS under fluorescence (2) or white light (6); KLE cells transfected with ShGPR30-pGFP-V-RS under fluorescence (3) or white light (7); KLE cells transfected with shiv-pGFP-V-RS under fluorescence (4) or white light (8). Magnification (1,5,2,6) ×200; (3,7,4,8) ×100. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)